Functional co-operation between the subunits in heterodimeric platelet-derived growth factor receptor complexes

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To determine the importance of the phosphorylation capacity of receptor kinase as well as the ability to serve as docking sites for SH2-domain-containing signal transduction molecules, we established pig aortic endothelial cell lines stably expressing kinase-active platelet-derived growth factor (PDGF) α -receptors together with kinase-inactive β -receptors, and vice versa. After stimulation with PDGF-AB, heterodimeric receptor complexes were formed in which the kinase-inactive receptor was phosphorylated by the kinase-active receptor, although less efficiently than in heterodimers of wild-type receptors. The kinase-active receptor was only minimally phosphorylated. Thus the phosphorylation within the receptor dimer occurred *in trans* between the components. Analyses of the abilities of

heterodimeric receptor complexes of one kinase-active and one kinase-inactive receptor to mediate mitogenicity, chemotaxis and activation of mitogen-activated protein kinase revealed less efficient effects than those of heterodimers of wild-type receptors. Importantly, however, the fact that signalling capacities were retained illustrates a functional co-operation between the two receptor molecules in the dimer, where one receptor provides a functional kinase and the other acts as a substrate and provides docking sites for downstream signalling molecules.

Key words: dimerization, endothelial cells, phosphorylation, signal transduction, tyrosine kinase receptor.

INTRODUCTION

Platelet-derived growth factor (PDGF) is important for cell growth, differentiation and motility and the survival of connective tissue cells and other cells; it has an important role during normal embryonal development (reviewed in [1]). PDGF isoforms are disulphide-bonded homodimeric or heterodimeric proteins composed of related A and B polypeptide chains. The diverse cellular effects of PDGF isoforms are mediated through binding with different specificities to two structurally related transmembrane receptors with intrinsic protein tyrosine kinase activity, denoted α - and β -receptors [1]. The β -receptor binds the B chain of PDGF, whereas the α -receptor binds both the A and B chains with high affinity. Thus PDGF-AA induces $\alpha\alpha$ receptor homodimers, PDGF-AB $\alpha\alpha$ homodimers and $\alpha\beta$ heterodimers, and PDGF-BB can induce all three possible combinations of dimers.

PDGF-induced receptor dimerization is followed by the autophosphorylation of certain tyrosine residues in the receptors, which has been proposed to occur *in trans* between the receptor molecules in the dimers [2]. Nine and twelve autophosphorylation sites have been identified so far in the α - and β -receptors respectively ([1], and L. Rönstrand, unpublished work). Auto-phosphorylated tyrosines located in the kinase domains regulate the catalytic activity of the kinases (Tyr-849 and Tyr-857 in the α - and β -receptors respectively), whereas other auto-phosphorylated residues serve as docking sites for SH2-domain-containing downstream signalling molecules. Thus a signalling complex is formed through ligand-induced receptor dimerization, and the complex activates multiple signal transduction pathways.

$\alpha\alpha$ and $\beta\beta$ receptor homodimers transduce overlapping but distinctly different cellular effects [3]. Moreover, the $\alpha\beta$ receptor

heterodimer seems to have unique properties that can be explained by the fact that the autophosphorylation of tyrosine residues occurs at different sites in the heterodimer from those in homodimeric receptor complexes. For example, Tyr-754 in the α -receptor becomes autophosphorylated to a high degree in the heterodimeric receptor complex [4]. Moreover, in the β -receptor, Tyr-771, the binding site for the Ras-inactivating protein RasGAP, is phosphorylated at a higher stoichiometry in the homodimer than in the heterodimer. This might explain the more efficient Ras activation and mitogenic stimulation by PDGF-AB, which preferentially induces $\alpha\beta$ heterodimeric complex, than by PDGF-BB, which induces all combinations of receptors [5].

Previous studies have shown that pig aortic endothelial (PAE) cells that express kinase-inactive β -receptor fail to elicit PDGF-BB-induced chemotaxis and actin reorganization [6]. However, kinase-inactive β -receptor can be phosphorylated by co-expressed wild-type α -receptor [2]. Moreover, whereas PDGF did not activate the mitogen-activated protein (MAP) kinase cascade in HepG2 cells in which the α -receptor was expressed at a low level, PDGF-BB-induced MAP kinase activation was observed after the transfection of kinase-inactive β -receptors; it was suggested that the mechanism involved phosphorylation of the kinase-inactive β -receptor by the α -receptor [7].

In the present study, kinase-inactive PDGF α -receptors (K627A mutant) and kinase-inactive β -receptors (K634A mutant) were co-expressed with wild-type receptors in PAE cells. The purpose of the study was to investigate the importance in signal transduction of the phosphorylation capacity of the active kinase and the ability of the kinase-inactive receptor to undergo autophosphorylation and thereby provide docking sites for SH2-domain-containing signalling molecules.

Abbreviations used: FBS, fetal bovine serum; MAP kinase, mitogen-activated protein kinase; PAE cells, pig aortic endothelial cells; PDGF, platelet-derived growth factor.

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MATERIALS AND METHODS

Plasmids and cell culture

With the use of the Altered sites *in vitro* mutagenesis system (Promega) site-directed mutagenesis was performed on cDNA encoding the human full-length PDGF α - and β -receptors [8,9]. The following oligonucleotides were used: 5'-GTTGCAGTGGCGATGCTAAAACCCACCGCCAGATCC-3' (K627A; α^-) and 5'-GTGGCCGTCGCTATGCTTAAATCC-3' (K634A; β^-). All mutations were confirmed by DNA sequencing. The mutated cDNA species were inserted into pcDNA3 (Invitrogen) and were co-transfected with pBABE/puro by electroporation into PAE cells expressing wild-type β -receptor (PAE/PDGFR- β 1.3) and wild-type α -receptor (PAE/PDGFR- α) respectively. Clones were selected for resistance to puromycin. Transient transfection of human wild-type or kinase-inactive α - or β -receptor into COS-1 cells was performed by the DEAE-dextran technique.

Cell clones expressing kinase-inactive α - and wild-type β -receptors were termed PAE/ $\alpha^- \beta$, and cell clones expressing wild-type α - and kinase-inactive β -receptors PAE/ $\alpha \beta^-$. Clones PAE/ $\alpha^- \beta$ 24 and PAE/ $\alpha \beta^-$ 9, with approximately equal amounts of α - and β -receptors, were used in the present study.

Antisera and reagents

The rabbit peptide antiserum PDGFR-3, recognizing the PDGF β -receptor, and antiserum PDGFR-7 against the PDGF α -receptor have been described previously [3,8]. A rabbit antiserum, EET, against Erk2 kinase was raised as described [10]. The monoclonal anti-phosphotyrosine antibodies PY20 and PY99 were purchased from Transduction Laboratories and from Santa Cruz Biotech respectively, peroxidase-conjugated pig anti-(rabbit Ig) and sheep anti-(mouse Ig) from Amersham, and myelin basic protein from Life Technologies-BRL. PDGF-AA, PDGF-BB and PDGF-AB were gifts from Amgen.

Immunoprecipitation and immunoblotting

Serum-starved cells were stimulated with different PDGF isoforms for 1 h on ice, then lysed and subjected to immunoprecipitation and immunoblotting essentially as described by Ekman et al. [5]. Lysates were also incubated with Wheat Germ Lectin Sepharose 6 MB (Pharmacia Biotech) for 30 min at 4 °C to enrich a glycoprotein fraction. Immunoprecipitates were heated at 95 °C for 3 min in reducing Laemmli sample buffer and separated by SDS/PAGE [7–12% (w/v) gel], followed by electrotransfer and immunoblotting as described by Ekman et al. [5].

Labelling with [³⁵S]methionine

For the identification of positive clones expressing kinase-inactive α -receptor or kinase-inactive β -receptor together with wild-type receptors (PAE/ $\alpha^- \beta$ or PAE/ $\alpha \beta^-$ cells respectively), cells were labelled with [³⁵S]methionine, followed by lysis and immunoprecipitation with antibodies specific for PDGF α - or β -receptors, essentially as described by Hansen et al. [11]. Samples were analysed by SDS/PAGE [7% (w/v) gel], followed by fluorography and exposure to X-ray film and/or Bio-Imager plates (Fuji).

MAP kinase assay

MAP kinase was assayed essentially by the method of Reuter et al. [12], with myelin basic protein as a substrate. The amount of

radioactivity incorporated into myelin basic protein was quantified with a Fuji BAS2000 Bioimager.

Chemotaxis assay

Chemotaxis was measured with a Boyden chamber by using Costar nucleopore filters coated with type I collagen, essentially as described by Kanda et al. [13]. The assays were performed in Ham's F-12 medium containing 0.3% BSA. In each experiment, the migration of upper chamber cells suspended in 0.3% BSA/Ham's F-12 medium towards 10% (v/v) fetal bovine serum (FBS) in 0.3% BSA/Ham's F-12 medium below the filter served as a control, and was referred to as 100% migration. Results are means $\pm$ S.D. for four experiments.

[³H]Thymidine incorporation assay

The thymidine incorporation assay was performed as described by Mori et al. [14], except that cells were starved in 0.3% FCS in Ham's F-12 medium before stimulation with ligand.

[³²P]P_i labelling, phosphopeptide mapping, Edman degradation and phosphoamino acid analysis

Procedures for labelling with [³²P]P_i, followed by stimulation of the cells with PDGF and immunoprecipitation of the wild-type and kinase-inactive α - and β -receptors, were performed essentially as described by Rönnstrand et al. [15]. Sequential immunoprecipitations were done, starting with the anti-phosphotyrosine antibody PY20. Immunoprecipitates were then boiled for 3 min in 100 μ l of 1% (w/v) SDS/10 mM dithiothreitol in TBS buffer [10 mM Tris/HCl (pH 7.5)/150 mM NaCl], and incubated at room temperature in 50 mM iodoacetamide (Sigma). The supernatant, containing the separated monomeric receptors, was transferred to a new tube, filled up to 1 ml with RIPA buffer [1% (v/v) Triton X-100/0.5% sodium deoxycholate/0.1% SDS/0.15 M NaCl/20 mM Tris/HCl (pH 7.4)/5 mM EDTA/30 mM Na₂P₂O₇/1% Trasylol/1 mM PMSF/200 μ M Na₃VO₄/50 mM NaF/5 μ g/ml leupeptin/1 mM dithiothreitol], then subjected to consecutive immunoprecipitations first with PDGFR-7 (recognizing PDGF α -receptor) and then with PDGFR-3 (recognizing PDGF β -receptor). The samples were subjected to SDS/PAGE, transferred to a nitrocellulose membrane and detected by autoradiography. The phosphorylated wild-type and kinase-inactive receptor bands were excised from the membrane and subjected to digestion with trypsin and two-dimensional phosphopeptide mapping, essentially as described by Blume-Jensen et al. [16].

RESULTS

PDGF-induced tyrosine phosphorylation of heterodimeric receptor complexes

To analyse autophosphorylation in heterodimeric complexes of kinase-inactive and wild-type PDGF receptors, PAE/ $\alpha^- \beta$ and PAE/ $\alpha \beta^-$ cells starved of serum overnight, as well as PAE/ $\alpha \beta$ cells for comparison, were treated or not with PDGF isoforms for 1 h on ice. The glycoprotein fraction of cell lysates was then enriched by adsorption to Wheat Germ Lectin Sepharose, separated by SDS/PAGE and subjected to immunoblotting with an anti-phosphotyrosine antibody, PY99. The α - and β -receptors have sizes of 170 and 180 kDa respectively and can be distinguished in the gel by their different mobilities (Figure 1, lanes 13 and 14). As shown in Figure 1, receptors from non-stimulated cells were not phosphorylated on tyrosine residues (lanes 1, 5 and 9). In PAE/ $\alpha \beta$ cells, PDGF-AA induced the phosphorylation of

Figure 1 PDGF-induced tyrosine phosphorylation of wild-type and kinase-inactive α - and β -receptors expressed in different combinations in PAE cells

PAE cells starved overnight in 0.3% (v/v) FBS were left unstimulated (—) or stimulated with indicated PDGF isoforms (100 ng/ml) for 1 h on ice, then lysed. PDGF receptor complexes were immunoprecipitated with Wheat Germ Lectin Sepharose. Samples were separated by SDS/PAGE, transferred to nitrocellulose membrane and analysed by Western blotting with an anti-phosphotyrosine antibody, PY99. The positions of PDGF receptors are indicated at the right.

α -receptors only, as expected (Figure 1, lane 2), whereas PDGF-AB and -BB induced the phosphorylation of both α - and β -receptors (lanes 3 and 4). In PAE/ $\alpha\beta$ cells, PDGF-AA did not induce the phosphorylation of kinase-inactive α -receptors, as expected (Figure 1, lane 6); PDGF-BB induced the phosphorylation of both α - and β -receptors, presumably in hetero- and homodimeric complexes respectively (lane 7), and PDGF-AB induced the phosphorylation of the kinase-inactive α -receptor only, presumably in a heterodimeric complex with the β -receptor (lane 8). In PAE/ $\alpha\beta$ cells, PDGF-AA induced increased α -receptor autophosphorylation, as expected, whereas both PDGF-BB and -AB induced the autophosphorylation of both α - and β -receptors (Figure 1, lanes 10, 11 and 12). The phosphorylation of the kinase-inactive β -receptor probably occurred in a heterodimer with the α -receptor; the α -receptor phosphorylation might have occurred in α -receptor homodimers, which are induced by both PDGF-AB and PDGF-BB.

These observations are all consistent with the notion that ligand-induced receptor dimerization induces autophosphorylation *in trans* in the receptor complex, consistent with the suggestion of Kelly et al. [2].

Cell migration

In PAE cells, the PDGF β -receptor mediates PDGF-induced cell migration, whereas the α -receptor has an inhibitory effect, when analysed with a thick-filter assay [17]. In the present study we evaluated the migratory response of PAE/ $\alpha\beta$, PAE/ $\alpha\beta$ and PAE/ $\alpha\beta$ cells, as well as that of cells expressing either the wild-type α - or β -receptors, towards different concentrations of PDGF isoforms by using a 48-well modified Boyden chamber apparatus with thin filters. The migration of upper-chamber cells, suspended in 0.3% BSA in Ham's F-12 medium towards the same medium containing 10% FBS below the filter, served as a control and was referred to as 100% migration. Unlike the findings with the thick-filter assay, we observed dose-dependent migration of both wild-type α - and wild-type β -receptor-expressing PAE cells on stimulation with appropriate PDGF isoforms (results not shown).

PAE/ $\alpha\beta$ cells, which express wild-type α - and β -receptors, responded to stimulation with 25–50 ng/ml PDGF-AB with a maximal migration of approx. 160% of that with 10% FBS (Figure 2A). In addition, PDGF-AA and PDGF-BB gave efficient stimulation, but with a maximum at higher concentrations. Stimulation of PAE/ $\alpha\beta$ cells with PDGF-BB or PDGF-AB resulted in migration, but only at approx. 50% of that with 10%

Figure 2 Migration of PAE cells, expressing different combinations of wild-type and kinase-inactive α - and β -receptors, towards different concentrations of PDGF-AA (●), PDGF-BB (■) and PDGF-AB (▲) in a modified Boyden chamber apparatus through a thin filter

The indicated concentrations of PDGF, diluted in 0.3% BSA/Ham's F-12 medium, were placed in the lower chamber. Approx. 50 000 cells suspended in 0.3% BSA/Ham's F-12 medium were placed in the upper chamber and incubated in a humidified incubator at 37 °C under air/CO₂ (19:1) for 4 h. The number of cells that migrated to the lower surface of the thin filter was determined. In each experiment, the migration of cells towards 10% (v/v) FBS in 0.3% BSA/Ham's F-12 medium served as a control and was referred to as 100% migration. Results are means $\pm$ S.D. for four experiments. (A) PAE/ $\alpha\beta$ cells; (B) PAE/ $\alpha\beta$ cells; (C) PAE/ $\alpha\beta$ cells.

FBS. The maximum effect was obtained at lower concentrations of PDGF-BB than of PDGF-AB. PDGF-AA had only a minimal effect (26%) (Figure 2B). PAE/ $\alpha\beta$ cells showed maximal migration towards the PDGF isoforms at relatively low concentrations of PDGF-AA and PDGF-AB (10 ng/ml) compared with PDGF-BB (25 ng/ml). The response to PDGF-AA and PDGF-BB was 70–80% of that to 10% FBS, but that to PDGF-AB was only approx. 45% (Figure 2C).

Thus PDGF-AB, which induces heterodimeric receptor complexes, had a more potent stimulatory effect on migration than PDGF-AA or PDGF-BB for PAE/ $\alpha\beta$ cells containing wild-type

Figure 3 Stimulation of [^3H]thymidine incorporation by PDGF isoforms in PAE cells expressing different combinations of wild-type and kinase-inactive α - and β -receptors

After 44 h of starvation in 0.3% (v/v) FBS, PAE/ $\alpha\beta$ cells (**A**), PAE/ $\alpha\beta^-$ cells (**B**) and PAE/ $\alpha\beta^-$ cells (**C**) were incubated with [^3H]thymidine and the indicated concentrations of PDGF-AA ($\bullet$), PDGF-BB ($\blacksquare$) and PDGF-AB ($\blacktriangle$) or 10% FBS for 24 h. Trichloroacetic acid-precipitated radioactivity was measured as described in the Materials and methods section. [^3H]Thymidine incorporation into cells incubated with 10% FBS was set at 100%; results are means $\pm$ S.D. for three experiments.

α - and β -receptors. PDGF-AB had a smaller stimulatory effect for PAE/ $\alpha\beta$ cells and PAE/ $\alpha\beta^-$ cells; thus heterodimer receptor complexes containing one kinase-inactive receptor mediated the stimulation of migration, although less efficiently than heterodimers of wild-type receptors.

Mitogenicity

Both PDGF α - and β -receptors are able to induce mitogenicity [3]. We investigated the abilities of different PDGF isoforms to stimulate DNA synthesis, measured as the incorporation of [^3H]thymidine, in PAE cells with different combinations of wild-type and kinase-inactive receptors. As shown in Figure 3(A), PDGF-AB had a stronger mitogenic effect than PDGF-BB or

PDGF-AA in PAE cells expressing wild-type α - and β -receptors, which is consistent with previous observations [4,5]. For PAE/ $\alpha\beta^-$ cells, PDGF-BB had a stronger mitogenic effect than PDGF-AB, whereas PDGF-AA, as expected, had no effect (Figure 3B). In PAE/ $\alpha\beta^-$ cells, the dose-dependent responses of PDGF-AA and -AB were comparable, whereas PDGF-BB yielded a lower stimulatory effect (Figure 3C).

These observations indicate that heterodimers in which a receptor is kinase-inactive ($\alpha\beta^-$ or $\alpha^-\beta$) transduce mitogenic signals but do so less efficiently than wild-type heterodimers ($\alpha\beta$). Thus the results of the mitogenicity assay are analogous to those of the migration assay.

MAP-kinase assay

The activation of the small GTP-binding protein Ras, and the subsequent activation of the MAP kinase cascade, is of major importance for mitogenic signalling mediated by receptor tyrosine kinases [18]. We therefore examined the ability of wild-type and kinase-inactive α - and β -receptor combinations to induce MAP kinase activation.

PAE/ $\alpha\beta$, PAE/ $\alpha\beta^-$ and PAE/ $\alpha\beta^-$ cells starved of serum overnight were treated with PDGF isoforms for different durations at 37 °C and were then lysed. The lysates were immunoprecipitated with an antiserum, EET, recognizing Erk2. The immunoprecipitates were subjected to a kinase assay with myelin basic protein as an exogenous substrate. In PAE/ $\alpha\beta$ cells, maximum activation of MAP kinase occurred after 5 min of stimulation; PDGF-AB induced a stronger activation of MAP kinase activity than PDGF-BB or PDGF-AA (Figure 4A). In contrast, in PAE/ $\alpha\beta^-$ cells, maximum activation occurred somewhat later, after 15 min, and PDGF-BB gave a more potent activation than PDGF-AB; PDGF-AA, as expected, had no effect on the activation of MAP kinase in these cells (Figure 4B). In PAE/ $\alpha\beta^-$ cells, PDGF-AA induced a stronger activation than PDGF-AB or PDGF-BB (Figure 4C). Thus in the MAP kinase assay, too, dimers in which one receptor was kinase-inactive gave lower stimulatory effects than dimers of kinase-active receptors.

[^{32}P]P_i labelling, phosphopeptide mapping and phosphoamino acid analysis

To determine whether phosphorylation sites were gained or lost in the wild-type or kinase-inactive α - and β -receptors in different dimeric combinations, PAE/PDGF- α , PAE/PDGF- β , PAE/ $\alpha\beta$, PAE/ $\alpha\beta^-$ and PAE/ $\alpha\beta^-$ cells were starved overnight in 0.3% FBS/Ham's F-12 medium, labelled with [^{32}P]P_i for 4 h and treated with PDGF-AB or -BB; PDGF receptors were then immunoprecipitated with anti-phosphotyrosine antibodies, then dissociated and immunoprecipitated again sequentially with antiserum against the α -receptors followed by antiserum against the β -receptors. After digestion with trypsin, samples were subjected to two-dimensional phosphopeptide mapping. Tryptic phosphopeptide maps of wild-type and kinase-inactive α -receptors, as well as of wild-type and kinase-inactive β -receptors expressed in different combinations, were compared.

Figure 5(A) shows the phosphopeptide pattern of wild-type α -receptor from PDGF-BB-stimulated PAE/PDGF- α cells. One heterodimer-specific weak phosphorylation site appeared in wild-type α -receptor from PDGF-AB-stimulated PAE/ $\alpha\beta$ cells (Figure 5B, filled arrowhead). Except for the gain of a phosphopeptide (Figure 5C, open arrowhead), kinase-inactive α -receptor from PDGF-AB-stimulated PAE/ $\alpha\beta^-$ cells had a similar phosphopeptide pattern to that of wild-type α -receptor (Figure 5C). Thus the wild-type and kinase-inactive α -receptors have very similar

Figure 4 PDGF-dependent MAP kinase activation in PAE cells expressing different combinations of wild-type and kinase-inactive receptors

Cells starved overnight in 0.3% (v/v) FBS were left unstimulated (control) or stimulated with 100 ng/ml of PDGF-AA (●), PDGF-BB (■) or PDGF-AB (▲) at 37 °C for the indicated durations. The preparation of cell lysates, immunoprecipitation of MAP kinase, assay for MAP kinase activity and quantification were as described in the Materials and methods section. Similar results were obtained in at least two individual experiments. (A) PAE/ $\alpha\beta$ cells; (B) PAE/ $\alpha\beta^-$ cells; (C) PAE/ $\alpha\beta^-$ cells.

phosphopeptide features. The major autophosphorylation site in the α -receptor, Tyr-849 (S. Ekman, C.-H. Heldin and L. Rönstrand, unpublished work), is marked with an asterisk in Figures 5(A)–5(C).

Figure 5(D) shows the phosphorylation pattern of homodimeric wild-type β -receptor from PDGF-BB-stimulated PAE/PDGFR- β cells. One heterodimer-specific prominent new phosphorylation site appeared in wild-type β -receptors from PDGF-AB-stimulated PAE/ $\alpha\beta$ cells (Figure 5E, filled arrowhead). The kinase-inactive β -receptor from PDGF-AB-stimulated PAE/ $\alpha\beta^-$ cells had similar phosphopeptide patterns to those of the wild-type β -receptors of PAE/ $\alpha\beta$ cells (Figure 5F). The major phosphorylation site in the β -receptor, Tyr-857 [19], is marked with a # symbol in Figures 5(D)–5(F).

Figure 5 Two-dimensional tryptic phosphopeptide mapping of the wild-type and kinase-inactive α - and β -receptors, expressed alone or in different combinations in PAE cells

PAE cells starved of serum overnight were labelled *in vivo* with [32 P] P_i , stimulated with PDGF-BB (A, D) or PDGF-AB (B, C, E, F), lysed in RIPA buffer, then consecutively immunoprecipitated with the anti-phosphotyrosine antibody PY20, anti-(α -receptor) antiserum PDGFR-7 and anti-(β -receptor) antiserum PDGFR-3. The immunoprecipitates were subjected to SDS/PAGE and transferred to nitrocellulose membranes. Wild-type and kinase-inactive α - and β -receptor bands were cut out and digested *in situ* with trypsin. The resulting digests were separated electrophoretically on TLC plates at pH 1.9, followed by ascending chromatography. Radioactive phosphopeptides were detected by autoradiography. (A) Wild-type α -receptor from PAE/PDGFR- α cells; (B) wild-type α -receptor from PAE/ $\alpha\beta$ cells; (C) kinase-inactive α -receptor from PAE/ $\alpha\beta^-$ cells; (D) wild-type β -receptor from PAE/PDGFR- β cells; (E) wild-type β -receptor from PAE/ $\alpha\beta$ cells; (F) kinase-inactive β -receptor from PAE/ $\alpha\beta^-$ cells. Symbols: *, Tyr-849-containing peptide from the α -receptor; #, Tyr-857-containing peptide from the β -receptor; filled arrowheads, heterodimer-specific phosphopeptides; open arrowhead, phosphopeptide specific for kinase-inactive α -receptor; arrows, sample application points.

DISCUSSION

The activation of tyrosine kinase receptors involves ligand-induced receptor dimerization. According to the prevalent hypothesis, the receptors thus come close enough to phosphorylate each other *in trans*. The results of the present study are fully compatible with this hypothesis; after dimerization of a kinase-active and a kinase-inactive PDGF receptor the kinase-inactive receptor becomes phosphorylated, whereas the kinase-active receptor is phosphorylated only to a minimal extent. The total amount of phosphorylation in a dimeric complex of one kinase-

active and one kinase-inactive receptor was found to be lower than in a dimer of kinase-active receptors. The lower degree of phosphorylation is correlated with the less efficient signalling to mitogenicity and migration that was observed.

The phosphorylation of a conserved tyrosine residue inside the kinase domain, Tyr-849 in the α -receptor and Tyr-857 in the β -receptor, has been shown to lead to increased catalytic activity of the kinase for several tyrosine kinases [20–25]. One explanation for the lower phosphorylation in a complex of kinase-active and kinase-inactive PDGF receptors might be that the phosphorylation of this critical amino acid residue occurs inefficiently and so the kinase domain will not be in a fully active configuration.

Autophosphorylation within the ligand-induced dimer of tyrosine kinase receptors is a key event in signal transduction, because it both leads to the activation of the kinase activity of the receptors and provides docking sites for downstream signal transduction molecules containing SH2 domains. An important conclusion of the present study is that the two receptors in the PDGF-induced signalling complex can co-operate functionally; one receptor might provide an active kinase and the other might provide docking sites for downstream SH2-domain-containing signalling molecules. There are examples of naturally occurring kinase-like receptors lacking catalytic activity, e.g. ErbB3 [26] and CCK4 [27]; after heteromerization with kinase-active receptors, such components might act as substrates and provide docking sites for downstream signalling molecules. An interesting question is whether the substrate specificities of the kinases of PDGF α - and β -receptors or the abilities to provide docking sites for SH2-domain-containing proteins are most important for signal specificity and efficiency. The cell lines described in the present paper are ideal in answering this question, provided that suitable cellular effects specific for α - and β -receptors respectively can be identified.

In agreement with previous studies [4,5], PDGF-AB was found to be more efficient than PDGF-AA or PDGF-BB in stimulating mitogenicity and chemotaxis in PAE/ $\alpha\beta$ cells. Although the reason for this finding remains to be fully elucidated, the occurrence of novel autophosphorylation sites might contribute. The characterization of such heteromer-specific autophosphorylation sites is currently continuing in our laboratory. Because the heterodimer-specific autophosphorylations observed in α - and β -receptors from an $\alpha\beta$ receptor heterodimer were seen also in $\alpha\beta$ and $\alpha\beta$ receptor heterodimers, it is likely that these differences in autophosphorylation are due to differences in specificity of the α - and β -receptor kinases. Alternatively, it is possible that α - and β -receptors show a differential association with highly specific protein tyrosine phosphatases, which causes the dephosphorylation of certain phosphorylation sites but not others. Interestingly, an additional phosphopeptide was observed in the kinase-inactive α -receptor compared with the kinase-active α -receptor after phosphorylation by the β -receptor. It is possible that this difference is due to the recruitment of a specific phosphatase to the autophosphorylated β -receptor in $\alpha\beta$ receptor complexes that is not recruited to the less phosphorylated β -receptor in $\alpha\beta$ receptor complexes.

The overexpression of kinase-inactive tyrosine kinase receptors is often used to achieve the inhibition of signalling through a

dominant-negative effect. The results of the present study illustrate that caution must be used when dominant-negative mutants of protein tyrosine kinase receptors are constructed, to avoid the possibility that kinase-inactive mutants serve as substrates for wild-type receptors and thereby act as docking sites for signalling molecules.

We thank Ivan Dikic for valuable comments on the manuscript, and Ingegärd Schiller for valuable secretarial assistance. This study was supported in part by a grant from the Axel and Margaret Ax:son Johnson Foundation.

REFERENCES

- Heldin, C.-H., Östman, A. and Rönstrand, L. (1998) *Biochim. Biophys. Acta* **1378**, F79–F113
- Kelly, J. D., Haldeman, B. A., Grant, F. J., Murray, M. J., Seifert, R. A., Bowen-Pope, D. F., Cooper, J. A. and Kazanietz, A. (1991) *J. Biol. Chem.* **266**, 8987–8992
- Eriksson, A., Siegbahn, A., Westermark, B., Heldin, C.-H. and Claesson-Welsh, L. (1992) *EMBO J.* **11**, 543–550
- Rupp, E., Siegbahn, A., Rönstrand, L., Wernstedt, C., Claesson-Welsh, L. and Heldin, C.-H. (1994) *Eur. J. Biochem.* **225**, 29–41
- Ekman, S., Rupp Thuresson, E., Heldin, C.-H. and Rönstrand, L. (1999) *Oncogene* **18**, 2481–2488
- Westermark, B., Siegbahn, A., Heldin, C.-H. and Claesson-Welsh, L. (1990) *Proc. Natl. Acad. Sci. U.S.A.* **87**, 128–132
- Vaillancourt, R. R., Gardner, A. M., Kazanietz, A. and Johnson, G. L. (1996) *Oncogene* **13**, 151–159
- Claesson-Welsh, L., Eriksson, A., Morén, A., Severinsson, L., Ek, B., Östman, A., Betsholtz, C. and Heldin, C.-H. (1988) *Mol. Cell. Biol.* **8**, 3476–3486
- Claesson-Welsh, L., Eriksson, A., Westermark, B. and Heldin, C.-H. (1989) *Proc. Natl. Acad. Sci. U.S.A.* **86**, 4917–4921
- Leekers, S. J. and Marshall, C. J. (1992) *EMBO J.* **11**, 569–574
- Hansen, K., Johnell, M., Siegbahn, A., Rorsman, C., Engström, U., Wernstedt, C., Heldin, C.-H. and Rönstrand, L. (1996) *EMBO J.* **15**, 5299–5313
- Reuter, C. W. M., Catling, A. D. and Weber, M. J. (1995) *Methods Enzymol.* **255**, 245–256
- Kanda, S., Landgren, E., Ljungström, M. and Claesson-Welsh, L. (1996) *Cell Growth Differ.* **7**, 383–395
- Mori, S., Claesson-Welsh, L. and Heldin, C.-H. (1991) *J. Biol. Chem.* **266**, 21158–21164
- Rönstrand, L., Mori, S., Arvidsson, A.-K., Eriksson, A., Wernstedt, C., Hellman, U., Claesson-Welsh, L. and Heldin, C.-H. (1992) *EMBO J.* **11**, 3911–3919
- Blume-Jensen, P., Wernstedt, C., Heldin, C.-H. and Rönstrand, L. (1995) *J. Biol. Chem.* **270**, 14192–14200
- Siegbahn, A., Hammacher, A., Westermark, B. and Heldin, C.-H. (1990) *J. Clin. Invest.* **85**, 916–920
- Seeger, R. and Krebs, E. G. (1995) *FASEB J.* **9**, 726–735
- Kazanietz, A. and Cooper, J. A. (1989) *Cell* **58**, 1121–1133
- Kazanietz, A., Durden, D. L. and Cooper, J. A. (1991) *Cell Regul.* **2**, 413–425
- Longati, P., Bardelli, A., Ponzetto, C., Naldini, L. and Comoglio, P. M. (1994) *Oncogene* **9**, 49–57
- Mitra, G. (1991) *Oncogene* **6**, 2237–2241
- Mohammadi, M., Dikic, I., Sorokin, A., Burgess, W. H., Jaye, M. and Schlessinger, J. (1996) *Mol. Cell. Biol.* **16**, 977–989
- Kato, H., Faria, T. N., Stannard, B., Roberts, Jr., C. T. and LeRoith, D. (1994) *Mol. Endocrinol.* **8**, 40–50
- Ellis, L., Clauser, E., Morgan, D. O., Ederly, M., Roth, R. A. and Rutter, W. J. (1986) *Cell* **45**, 721–732
- Guy, C. T., Muthuswamy, S. K., Cardiff, R. D., Soriano, P. and Muller, W. J. (1994) *Genes Dev.* **8**, 23–32
- Mossie, K., Jallat, B., Alves, F., Sures, I., Plowman, G. D. and Ullrich, A. (1995) *Oncogene* **11**, 2179–2184